#18
GND

VANMA48.001APC

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Lasb et al.	Group Art Unit 1644
Appl. No.	:	08/765,837	
Filed	:	September 7, 1999	
For	:	ANTIGENIC POLYPEPTIDE SEQUENCE OF FACTOR VIII, FRAGMENTS AND/OR EPITOPES OF THIS SEQUENCE	
Examiner	:	P. Nolan	

I hereby certify that this correspondence and all attached attachments are being deposited with the United States Postal Service as first-class mail in an envelope addressed to: United States Patent and Trademark Office, P.O. Box xxxx, Arlington, VA xxxxx, on

May 6 2002
(Date)

Daniel Hunt
David Hunt, Reg. No. 40,437

DECLARATION UNDER 37 C.F.R. §1.132

United States Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

1. This Declaration is being submitted to demonstrate that the linear Factor VIII peptides which we identified as having a high likelihood of being antigenic are in fact antigenic, that antibodies can be generated against the linear Factor VIII peptides in rabbits, that antibodies which recognize the linear Factor VIII peptides are present in human serum, and that antibodies which recognize the linear Factor VIII peptides inhibit Factor VIII activity.

2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.

3. I have extensive experience in the field of immunology and molecular biology as evidenced by my attached curriculum vitae (Exhibit A).

4. Identification of Peptides with a High Probability of Antigenicity

The peptides of the present invention were identified using molecular modeling and characterization of surface regions of Factor VIII (FVIII) to identify peptides with a high probability of antigenicity. Using the 3-D molecular model of the A domains built by Pemberton et al (1997), FVIII-peptide epitopes identified by the Parker and Hodge algorithms were located. As predicted by these algorithms, all peptides located in the A domains were found on the FVIII surface and were fully accessible to specific antibodies. Then, more than 30 surface regions (linear epitopes) spanning 8 to 25 residues characterized by a high hydrophilicity, flexibility and accessibility were identified on the FVIII molecule. Figures 2 through 5 of the specification of the above-identified application as filed reveal the results of the characterization.

On the basis of the high probability that they are located at the surface of the FVIII molecule (see Fig. 1 of the above-identified application for A3), linear peptides (P7 to P9) were selected, matching identified stretches of 13 or more amino-acid residues.

5. Immunogenicity of Factor VIII Peptides in Rabbits.

The ability of Factor VIII peptides identified as above to elicit an immune response in rabbits and the ability of antibodies against the peptides to inhibit Factor VIII activity was determined as follows. The following peptides were synthesized:

SEQ ID 01: the epitope arginine 1652 to tyrosine 1664 inclusive, defined by the following sequence: Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr ,

SEQ ID 02:the epitope aspartic acid 1681 to arginine 1696 inclusive, defined by the following sequence: Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg , and

SEQ ID 05: the epitope glutamic acid 1794 to tyrosine 1815 inclusive, defined by the following sequence: Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr .

The peptides were coupled to ovalbumin for production of specific antiserum. A known Factor VIII antigen, the epitope described by Shima et al (1988), was used as an external control.

Two rabbits were immunized with the synthetic peptides (SEQ ID 01, SEQ ID 02 and SEQ ID 05) of the FVIII domains conjugated with ovalbumin and these conjugates were injected into rabbits. The resulting FVIII anti-peptide antisera (RAP7, RAP8 and RAP9, respectively) of the corresponding peptides were then further characterized as follows.

Specifically, the antisera titer was assayed by ELISA using rFVIII or FVIII-peptide-KLH as the antigen. ELISA titer is expressed as the negative log of the reciprocal of the serum dilution giving 50% binding. The results are provided in column c of Exhibit B. The binding reaction of each anti-FVIII-peptide antiserum was specific both for the FVIII peptide used to elicit the immune response in rabbit and for rFVIII.

To demonstrate the FVIII epitope specificity of the rabbit anti-peptide antibodies, rFVIII and the rFVIII fragments obtained after treatment with thrombin were resolved by SDS-PAGE and analyzed by western blotting with the different preparations of rabbit IgGs (RAP7 to RAP9 IgGs). The immunoglobulins were then purified by chromatography on peptide-bound Sepharose. The FVIII domain recognized by the anti-FVIII peptide Ig after immunoblotting is shown in column d of Exhibit B.

Ig protein recoveries were also measured using immunoglobulins as the standard. The results are shown in column e of Exhibit B.

The Factor VIII inhibitory activity, expressed in BU/mg protein, was determined in a FVIII neutralizing activity assay Factor VIII activity was determined in a one-stage clotting assay adapted for use on the Coagulometer KC4A (Sigma Diagnostics). The assay uses severe hemophilia A plasma (Organon Teknika, Cambridge, UK) and APTT reagent from Instrumentation Laboratory (Warrington, UK). Potencies were calculated relative to the 5th International Standard FVIII concentrate 88/640 (3.4 IU/ml) (NIBSC, Potters Bar, UK). FVIII-inhibitory activity was measured in purified rabbit IgG preparations according to the modified Bethesda assay. Briefly, affinity-purified IgGs were serially diluted and incubated for 1 h in the presence of FVIII concentrate 88/640 (1 IU/ml) at 37°C. The residual FVIII activity was measured as described above. The results are shown in column f of Exhibit B.

The results summarized in Exhibit B demonstrate the production of antibodies to peptides of the invention (column b, Exhibit B), corresponding to antigenic regions found in the A3 domain of the intact FVIII molecule (column d, Exhibit B). Assays performed with these antibodies demonstrate the inhibition of FVIII activity with the rabbit anti-(Arg^{1681}-Tyr^{1690}) antibodies at 0.5 BU/mg (column f, Exhibit B).

5. Immunogenicity of Factor VIII Peptides in Humans

The antigenicity of the Factor VIII peptides in humans was determined as follows. From a plasma pool of 4800 unpaid donors, Cohn fraction II+III, rich in immunoglobulins, was

analyzed for the presence of selected specific anti-peptide antibodies. Human anti-FVIII-peptide antibodies (HAP7 to 9) were purified by affinity chromatography on Sepharose coupled to the appropriate peptide (SEQ ID 01, SEQ ID 02 and SEQ ID 05, respectively). Exhibit C details the results of the diagnostic assays performed with the human anti-FVIII-peptide antibodies.

As illustrated in column b of Exhibit C, the human anti-peptide IgG preparations were analyzed by immunoblotting. Binding to the rFVIII HC or LC chains and to rFVIII thrombin proteolysis fragments is shown respectively in columns c and d, Exhibit C. The specificity of the resulting purified human antibodies was directly tested by immunoblotting with plasma FVIII, recombinant FVIII, and the fragments obtained after thrombin proteolysis. FVIII-domain reactivity is shown in column e, Exhibit C. Ig recovery (column f, Exhibit C) after affinity purification is expressed in µg/10 mg loaded FII+III. Inhibition of Factor VIII activity in a clotting assay was determined after incubation in the presence of each of the 3 Ig preparations in the Bethesda assay (column g, Exhibit C), as described above.

The summarized results in Exhibit C demonstrate the presence of antibodies to peptides of the invention (column b, Exhibit C), corresponding to antigenic regions found in the A2 domain of the intact FVIII molecule (column e, Exhibit C) in human serum. Results from the assay performed using human anti-FVIII peptide antibodies (Exhibit C, column g) indicate that inhibition of procoagulant activity was displayed in anti-(Asp^{481}-Arg^{496}) antibodies and anti-(Arg^{797}-Tyr^{812}) antibodies (HAP8, corresponding to SEQ ID 02 and HAP9, corresponding to SEQ ID 05, respectively).

Thus, the A2 peptides identified as described above were antigenic in humans and antibodies directed against them inhibited Factor VIII activity.

8. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 05/03/2002 By: _____
Ruth Lamb

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072901

VANMA48.001APC

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Laub et al.	Group Art Unit 1644
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May 6, 2002
(Date)

Daniel Hart, Reg. No. 40,637

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Thus, the A3 peptides identified as described above were antigenic in humans and antibodies directed against them inhibited Factor VIII activity.

8. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: _____ By: _____
Ruth Laub

SEQ ID NO:17:

Ser Cys Aso Lys Asn Thr Gly Asp Tyr Try Gly Asp Ser Tyr Glu Asp
1 5 10 15

13. Fragment antigénique de la séquence polypeptidique antigénique C selon la revendication 6, caractérisé en ce qu'il est compris entre la Lysine 2085 et la Lysine 2249, de préférence entre la Lysine 2085 et la Glycine 2121.

14. Epitope de séquence du fragment selon la revendication 13, caractérisé en ce qu'il est choisi parmi le groupe constitué par :

- l'épitope compris entre la Lysine 2085 et la Phénylalanine 2093 défini par la séquence suivante :

SEQ ID NO:18:

Lys Thr Gln Gly Ala Arg Gln Lys Phe
1 5

- l'épitope compris entre l'Acide Aspartique 2108 et la Glycine 2121 défini par la séquence suivante :

SEQ ID NO:19:

Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly
1 5 10

- l'épitope compris entre la Glycine 2242 et la Lysine 2249 défini par la séquence suivante :

SEQ ID NO:20:

Gly Val Thr Thr Gln Gly Val Lys
1 5

15. Partie forte des fragments et/ou des épitopes selon l'une quelconque des revendications précédentes 6 à 14, caractérisée en ce qu'elle comprend l'acide aminé Tyrosine et/ou l'acide aminé Histidine lié à au moins deux autres acides aminés identiques ou différents.

16. Epitope conformationnel caractérisé en ce qu'il comprend au moins deux fragments différents, au moins deux épitopes différents et/ou au moins deux parties fortes différentes selon l'une quelconque des revendications

EXHIBIT A

CURRICULUM VITAE

LAUB, Ruth

NATIONALITY
Belgian

UNIVERSITY TRAINING
PhD - Molecular Biology Department - Free University of Brussels

PROFESSIONAL ADDRESS
5, rue Joseph Stallaert, 1060 Brussels
Phone : + 32/2-349.69.11
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6, avenue Besme, 1190 Brussels
Phone & Fax : + 32/2-343.21.43

PRESENT FUNCTIONS
- R&D Manager - Central Department for Fractionation, Belgian Red Cross
- Guest Lecturer - Free University of Brussels, Centrum University of Charleroi
- Administration Board Member Belgian Biotechnology Association (BBA)
- WHO SoGAT participant

PREVIOUS FUNCTIONS
- Research Senior : International Institute of Cellular Pathology Brussels (ICP)
  (Specialization : immunology - microbiology cell physiology)
- Director Study : Institut des Industries de Fermentation - Ceria
  (Specialization : fermentation of recombinant strains and industrial process)

PUBLICATIONS
- In biochemistry and immmology.

PATENTS
- In biochemistry and pharmaceutical fields.

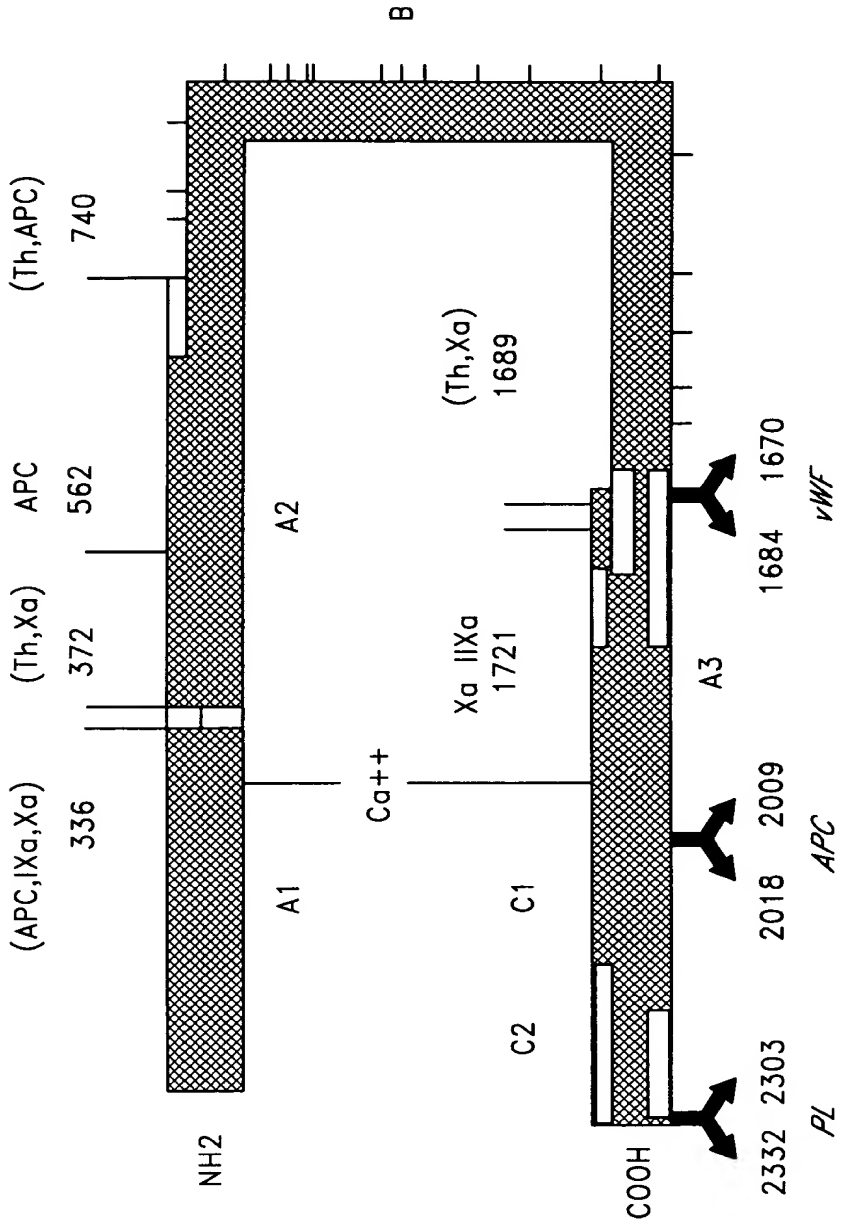
EXHIBIT B: **Table 1. Characterization of rabbit anti-FVIII peptides antibodies**

SEQ ID(a)	Rabbit Antiserum(b)	ELISA Titer(c)		FVIII domain recognize(d)	RAP-IgG Recovery(e) µg/ml serum	Inhibitor Titer(f) BU/mg
		P-KLH	r-FVIII			
SEQ ID 01 (Arg^{1652}-Tyr^{1664})	RAP7	3.9	3.9	a3 ↓	35	0.5
SEQ ID 02 (Asp^{1681}-Arg^{1696})	RAP8	1.9	0.9	a3/A3 ↓	3	-
SEQ ID 05 (Glu^{1794}-Tyr^{1815})	RAP9	3.8	2.6	A3	42	-

EXHIBIT C: **Table 2. Characterization of human anti-FVIII peptides autoantibodies**

SEQ ID(a)	Human Anti-peptide Ig(b)	FVIII reactivity on immunoblot (-thrombin)(c)	(+thrombin)(d)	FVIII domain(e)	HAP-IgG Recovery(f) µg/10 mgIgG	FVIII inhibitory Activity(g) BU/mg
SEQ ID 01 (Arg^{1652}-Tyr^{1664})	HAP7	80kDa	80kDa	a3 ↓	0.20	-
SEQ ID 02 (Asp^{1681}-Arg^{1696})	HAP8	80kDa	80kDa	a3/A3 ↓	0.01	+
SEQ ID 05 (Glu^{1794}-Tyr^{1815})	HAP9	80kDa	72kDa	A3	0.08	+

+: Inhibition >25% at 100µg/ml


B
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740
(Th,Xa)
1689
APC
562
A2
(Th,Xa)
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1721
1670
vWF
1684
A3
(APC,IXa,Xa)
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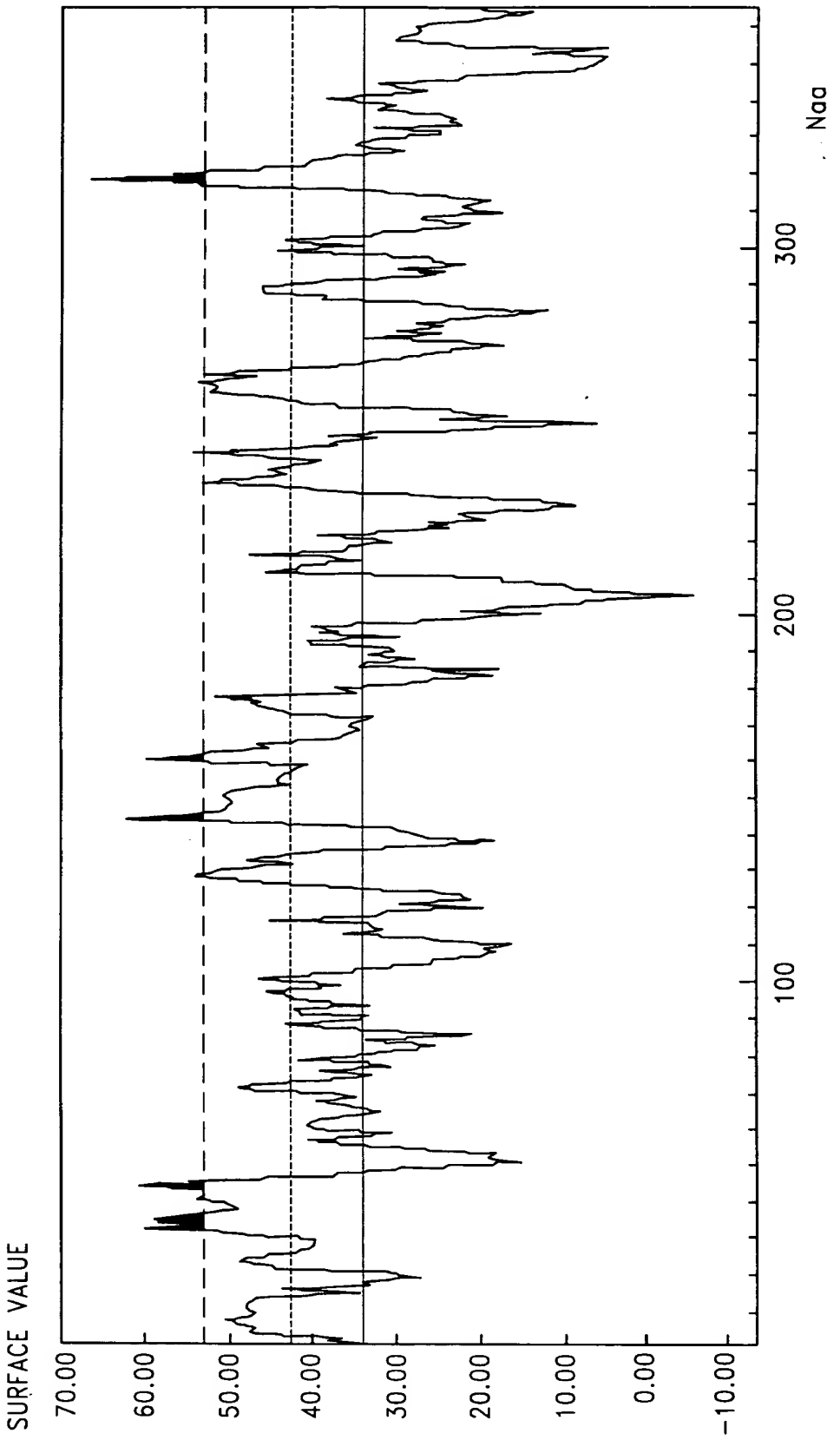
APC
A1
C1
2018
C2
2303
PL
NH2
COOH
2332

FIG. 1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Naa

FIG.2

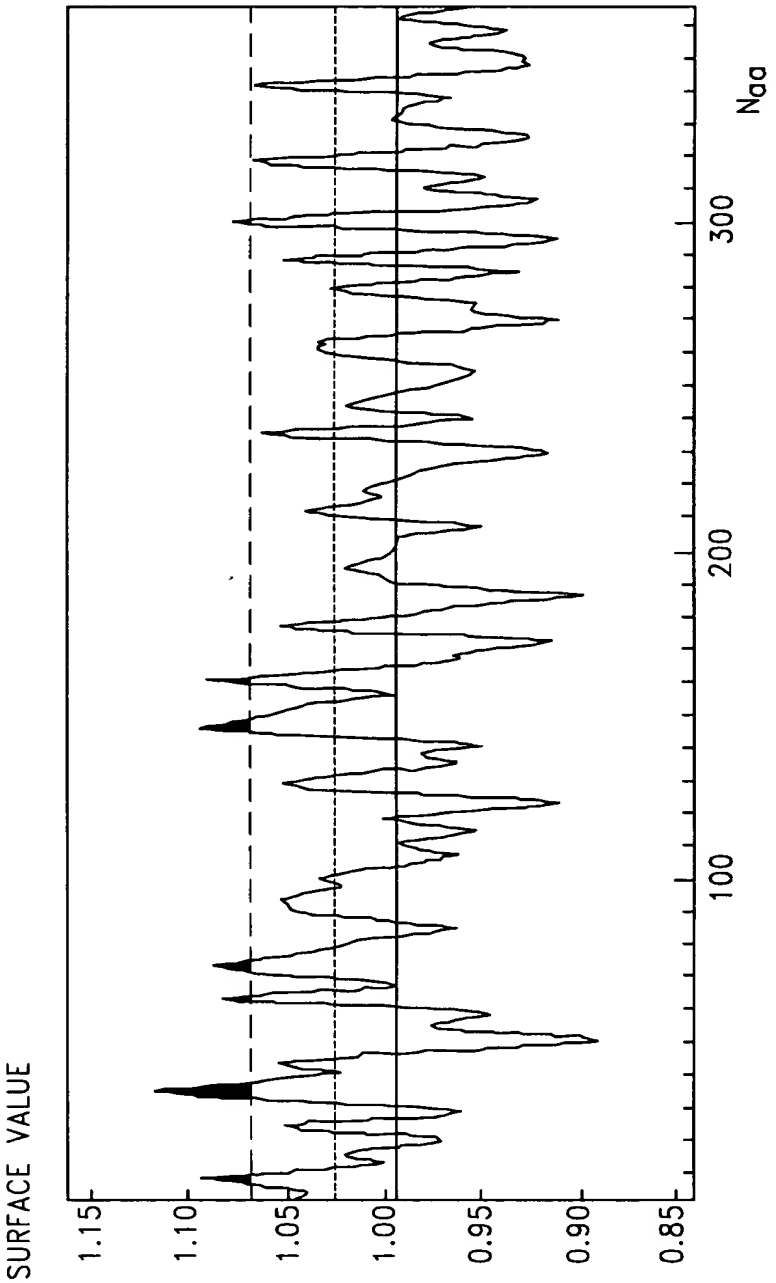
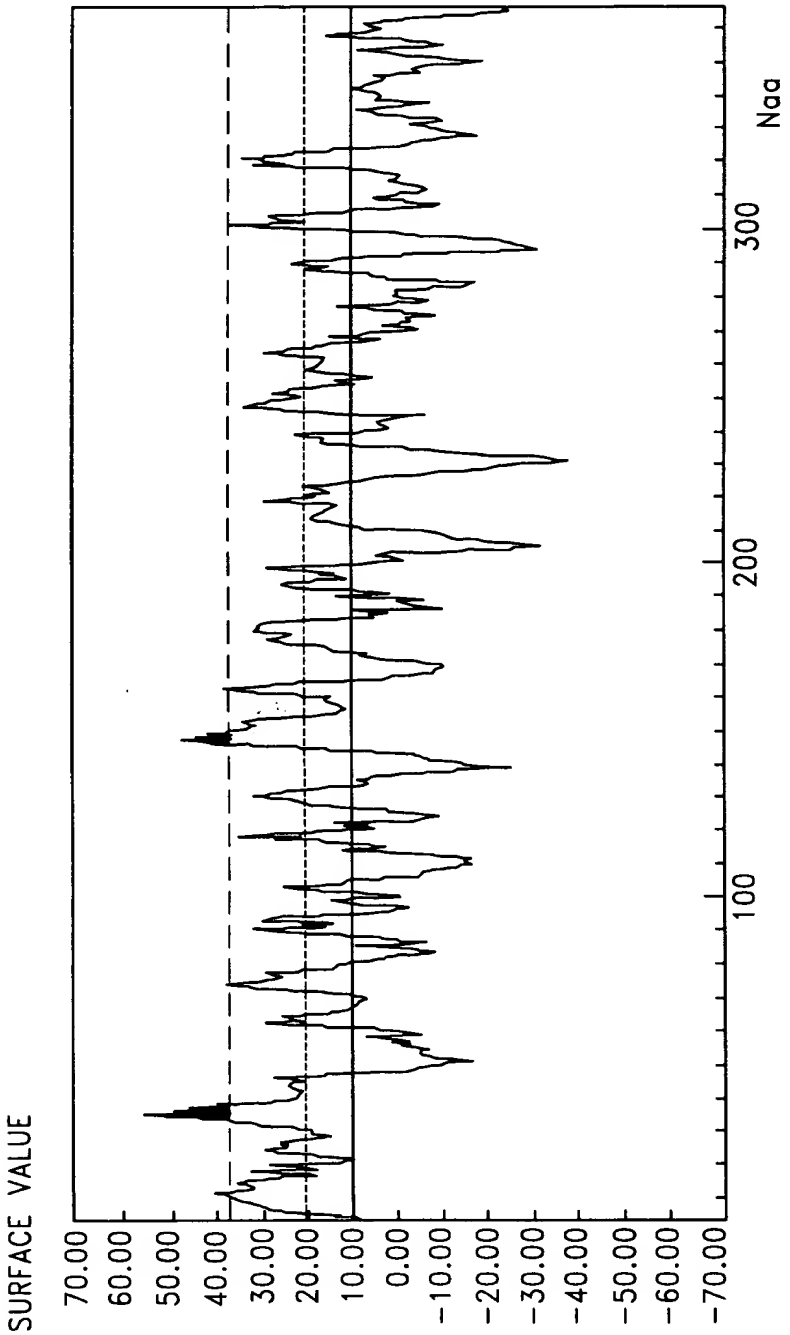


FIG.3


SURFACE VALUE
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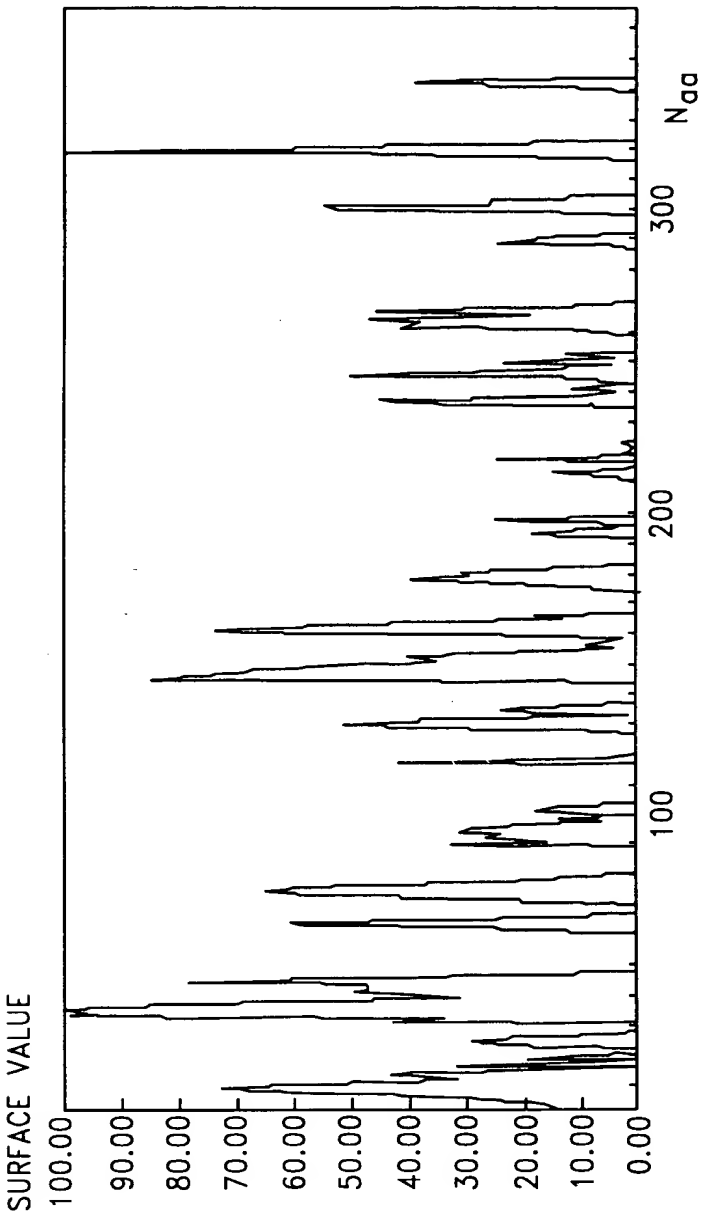
-60.00
-70.00
100
200
300
Naa

FIG. 4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0.00
100
200
300
Naa

FIG.5

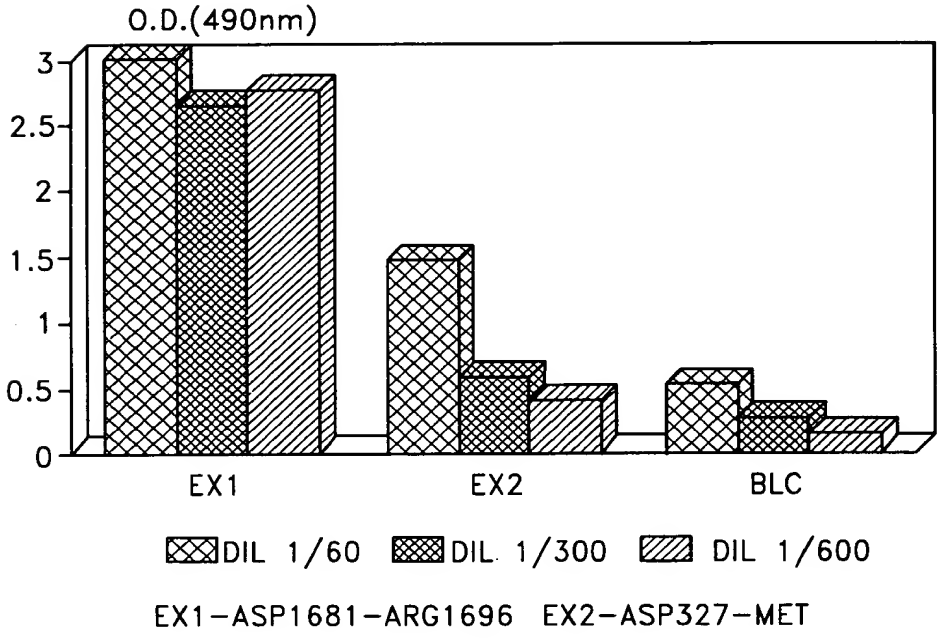
O.D.(490nm)
3
2.5
2
1.5
1
0.5
0
EX1
EX2
BLC
DIL 1/60
DIL 1/300
DIL 1/600
EX1-ASP1681-ARG1696 EX2-ASP327-MET

FIG.6